Biochemical and Biophysical Research Communications 458 (2015) 693–699

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Oregonin reduces lipid accumulation and proinflammatory responses in primary human macrophages

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ARTICLE INFO

Article history:
Received 12 January 2015
Available online 14 February 2015

Keywords:
Oregonin
Macrophages
Lipid accumulation
Cytokines
Anti-inflammatory

ABSTRACT

Inflammation in the vascular wall is important for the development of atherosclerosis. We have previously shown that inflammatory macrophages are more abundant in human atherosclerotic lesions than in healthy arteries. Activated macrophages produce reactive oxygen species (ROS) that promote local inflammation in atherosclerotic lesions. Here, we investigated the role of oregonin, a diarylheptanoid, on proinflammatory responses in primary human macrophages and found that oregonin decreased cellular lipid accumulation and proinflammatory cytokine secretion. We also found that oregonin decreased ROS production in macrophages. Additionally, we observed that treatment of lipopolysaccharide-exposed macrophages with oregonin significantly induced the expression of antioxidant-related genes, including *Heme oxygenase-1* and *NADPH dehydrogenase quinone 1*. In summary, we have shown that oregonin reduces lipid accumulation, inflammation and ROS production in primary human macrophages, indicating that oregonin has anti-inflammatory bioactivities.

1. Introduction

Macrophages that are derived from foam cells play integral roles in atherosclerosis and are key regulators of lipid-driven proinflammatory responses that promote atherosclerosis [1]. We have previously shown that ischemia induces lipid accumulation in primary human macrophages [2]. An inflammatory subset of macrophages accumulates in atherosclerotic lesions and produces proinflammatory cytokines, such as tumor necrosis factor (TNF)-α, interferon (IFN)-γ, interleukin (IL)-1β, and IL-6 [1,3]. Direct evidence for the critical role of inflammation in regulating early atherogenic events in atherosclerosis was provided by the finding that sub-endothelial monocytes could develop into foam cells after trans-endothelial migration across a TNF-α activated endothelium [4,5]. The numbers of proinflammatory macrophages have been shown to be increased in symptomatic compared with asymptomatic lesions [6]. Additionally, proinflammatory markers are expressed at higher levels in macrophages from human carotid lesions, suggesting a role for these cells in the atherosclerotic process. High levels of reactive oxygen species (ROS) in the artery wall are widely considered to promote atherosclerosis progression and possibly even initiation [7]. ROS are highly reactive molecules that are continuously produced by the mitochondrial electron transport chain and by ROS producing enzymes, such as NADPH-oxidases, xanthine oxidases, and lipoxygenases. At physiological levels, ROS perform important regulatory functions within cells and in tissues. At excessive levels, ROS irreversibly damage proteins, lipids, carbohydrates, and DNA. ROS levels have been reported to be high in stable atherosclerotic arteries, and such levels are even higher in unstable lesions [8].

Oregonin, an open-chain diarylheptanoid glycoside containing 3-carbonyl and 5-xylosyloxy groups, is found in the *Alnus* plants. Evidence indicates that this compound has anti-inflammatory and anti-oxidant properties. For example, *Alnus japonica* bark has been

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http://dx.doi.org/10.1016/j.bbrc.2015.01.161

used as a health food to enhance immunity against influenza [9]. Previous studies have shown that oregonin from *A. japonica* suppresses inflammation in rabbit macrophages by regulating NF-κB signaling [10]. Furthermore, other studies have shown that oregonin reduces the expression of adhesion molecules and IL-1β in TNF-α-stimulated human endothelial cells [11]. Moreover, studies have shown that *Alnus acuminata* bark extracts possess strong anti-inflammatory bioactivities [12]. However, little is known about the effects of oregonin from *Alnus incana* on lipid accumulation and anti-inflammatory responses in primary human macrophages.

Here, we investigated the effect of oregonin from *A. incana* on foam cell formation, inflammation, and ROS production in human monocyte-derived macrophages and found that oregonin decreases cellular lipid accumulation, ROS production, and the secretion of proinflammatory cytokines.

2. Materials and methods

2.1. Oregonin isolation

Bark from the grey alder *A. incana* was collected in southwestern Latvia. Freeze-dried and powdered (fine enough to pass through a 420-μm sieve) bark was sequentially extracted using a Soxhlet apparatus with solvents of increasing polarity (first hexane, then ethyl acetate). The open-chain diarylheptanoid, 1,7-bis-(3,4-dihydroxyphenyl)-heptan-3-one-5-O-β-D-xylopyronoside, known as oregonin (Fig. 1A), was isolated from the bark ethyl acetate extract to 95% purity using the Biotage (Charlottsville, VA, USA) SP1 preparative chromatography system with a reverse-phase column (KPC18-HS, 35–70 mm, 90A, Biotage) using aqueous ethanol [13].

2.2. Primary human macrophages

Buffy coats were obtained from volunteer healthy adult blood donors at Kungälv Hospital, Sweden, and samples were made anonymous before handling. Human mononuclear cells were isolated by centrifugation over a discontinuous gradient of Ficoll-Paque (GE Healthcare, Little Chalfont, UK). To obtain proinflammatory macrophages, isolated cells were seeded in sterile 6-well plates (TPP, Switzerland) at a cell density of 2×10^6 cells/mL in RPMI medium and incubated for 2 h at 37 °C in a 5% CO_2 incubator to allow adherence of monocytes. After washing off non-adherent cells, monocytes were cultured in Macrophage-SFM media (Gibco, Carlsbad, CA, USA) containing granulocyte macrophage colony stimulating factor (GM-CSF) to allow differentiation. After 3 days, the media was changed to RPMI media without GM-CSF and cells were cultured for 4 days before use in experiments.

2.3. Quantification of Oil Red O-stained lipid droplets

Primary human macrophages were seeded in 8-well chamber slides at a cell density of 400 000 cells/well (Lab-Tek Systems) as described above. The cells were incubated under ischemic conditions (1% oxygen) for 24 h, in the absence or presence of 50 μmol/L oregonin. This oregonin concentration is shown to lower cholesterol levels in rats with induced hypercholesterolemia [14]. Cells were stained with Oil Red O and hematoxylin, and viewed with a Zeiss Axioplan 2 microscope. We analysed20 randomly selected images, containing approximately 20 cells/image, for each condition and blinded quantification of the total area (μm^2) of Oil Red O-stained lipid droplets per cell was performed using BioPix iQ 2.2.1 (Gothenburg, Sweden; see www.biopix.se for further information) [15].

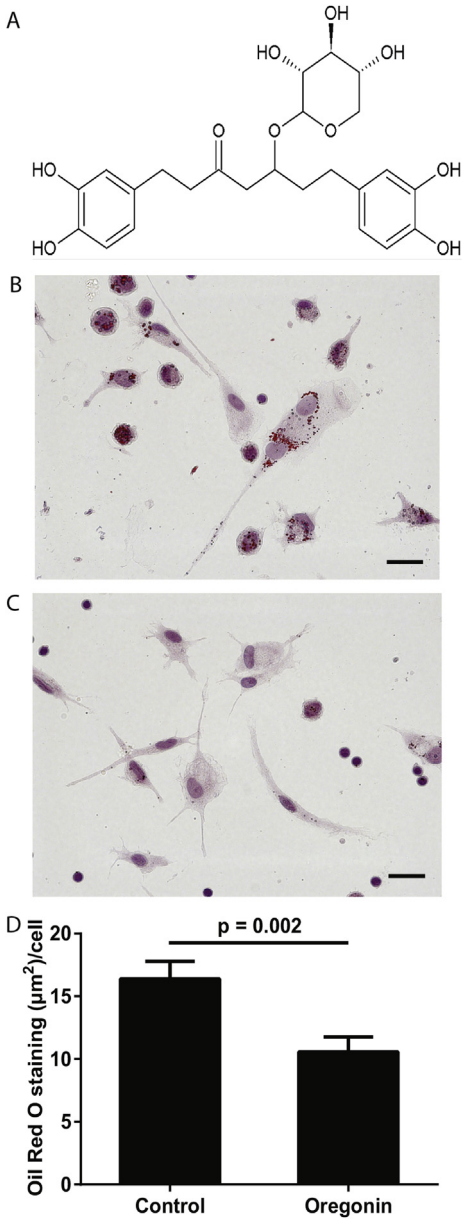

Fig. 1. Decreased cytosolic lipid droplets in oregonin-treated primary human macrophages. **A)** Oregonin, the open-chain diarylheptanoid, 1,7-bis-(3,4-dihydroxyphenyl)-heptan-3-one-5-O-β-D-xylopyronoside, was found to be the major bioactive compound in bark extracts from the grey alder (*Alnus incana*). **B–D)** Primary human macrophages were grown on chamber slides and incubated under ischemic conditions (1% oxygen) for 24 h, in the absence or presence of 50 μmol/L oregonin. Lipid accumulation was analyzed using Oil Red O staining. Representative images showing Oil Red O staining of **B)** control and **C)** oregonin-treated macrophages. **D)** Quantification of the total area (μm^2) of Oil Red O-stained lipid droplets per cell. Each bar indicates the mean ± SEM of all cells present in 20 randomly selected micrographs from four different donors. Bar 20 μm.

2.4. Cell viability

The cell viability was measured by using the Trypan blue exclusion test. Cells incubated with LPS or with oregonin in 6-well plate were cultured under normoxic conditions or hypoxic conditions for 24 h. Then the cells were washed with PBS and 0.5 mL of 0.2% trypan blue solution in PBS was added onto the cells and incubated for 5 min. Both the blue dead cells and living cells were counted.

2.5. Cytokine analyses

Primary human macrophages seeded in sterile 6-well plates as described above were incubated for 24 h, with or without 1 μg/mL lipopolysaccharide from *Salmonella enterica serotype typhimurium* (LPS; Sigma–Aldrich, St. Louis, MO, USA), in the absence or presence of 50 μmol/L oregonin in RPMI 1640 media. Secreted cytokines (GM-CSF, IFN-γ, IL-1β, IL-2, IL-6, IL-8, IL-10, IL-12p70, and TNF-α) were measured in supernatants from these cells using the Human Proinflammatory Multiplex assay (Meso Scale Discovery, Rockville, MD, USA; K15007C) according to the manufacturer's instructions and a SECTOR Imager 2400 reader (Meso Scale Discovery).

2.6. ROS measurements

Primary human macrophages were incubated for 24 h with or without 1 μg/mL LPS (Sigma), in the absence or presence of 50 μmol/L oregonin in RPMI 1640 media. Hydrogen peroxide (H_2O_2) levels were measured in lysates from 1×10^6 human macrophages using the Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen, Paisley, UK).

Superoxide anion production was measured using isoluminol-enhanced chemiluminescence (CL) [16]. The CL response was detected in 5×10^5 human macrophages incubated in the absence or presence of oregonin (50 μmol/L) in a total volume of 1.0 mL Krebs Ringer Glucose Buffer containing 10 μg isoluminol (Sigma), and 4 U horseradish peroxidase (Roche AB, Stockholm, Sweden). Signals were quantified using a luminescence counter (Bio Orbit Luminometer 1251, Turku, Finland). To activate the macrophages and induce ROS production, 5 nmol/L phorbol myristate acetate (PMA), a protein kinase C activator was added immediately prior to the measurements. Chemiluminescence was recorded for 20 min.

2.7. Quantitative PCR (q-PCR)

Primary human macrophages seeded in sterile 6-well plates as described above were incubated for 24 h, with or without 1 μg/mL lipopolysaccharide from *S. enterica* serotype typhimurium (LPS; Sigma–Aldrich, St. Louis, MO, USA), in the absence or presence of 50 μmol/L oregonin in RPMI 1640 media. RNA was isolated from these cells using a RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and cDNA was synthesized using the RT^2 First Strand Kit (C-03, SuperArray, SABiosciences, Fredrick, MD, USA). Reverse transcription reactions were performed with a Gene Amp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). To assess the expression of oxidative stress and antioxidant defense genes, the human PCR Array (PAHS-065ZA, Qiagen) were used. Real-time PCR amplification was performed for 40 cycles using an ABI Prism 7900 HT sequence detection system (Applied Biosystems). We analyzed q-PCR data using the comparative CT method [17]. The relative quantification of target gene mRNA expression levels was normalized to *HPRT1* mRNA expression.

2.8. Statistics

Values are mean ± SEM. All analyses were performed with GraphPad Prism version 6.0 for Windows (GraphPad Software). Student's t-test was used for comparison between two groups. Values of $P < 0.05$ were considered significant. The GeneGlobe Data Analysis Center (Qiagen) was used to analyze real-time PCR data based on the ΔΔCt method with normalization of the raw data to HPRT1 housekeeping gene as control and a criterion of fold change ≤1.5 to be considered as up- or down regulated.

3. Results

3.1. Oregonin treatment decreases the accumulation of cytosolic lipid droplets in human macrophages

To investigate the influence of oregonin on lipid droplet formation, human macrophages were incubated in ischemic conditions, which have previously been shown to increase cytosolic lipid droplets, in the absence or presence of oregonin. Exposure of human macrophages to oregonin decreased the pool of cytosolic lipid droplets, measured as the total area of Oil Red O-stained lipid droplets per cell (Fig. 1B–D).

3.2. Oregonin reduces cytokine production in LPS-activated human macrophages

We next tested the capacity of oregonin to reduce inflammation in macrophages that were activated by LPS. Oregonin significantly reduced levels of the inflammatory cytokines TNF-α, IL-1β, IL-2, IL-6, IL-10, and IL-12p70 in the supernatants of LPS-activated macrophages, whereas no changes were found in macrophages that were not treated with LPS (Fig. 2A–I). Analyses of GM-CSF, IFN-γ, and IL-8 levels showed no significant change after oregonin treatment (Fig. 2A, B and G). Exposure to 50 μmol/L oregonin did not significantly affect human macrophage viability, as measured by the trypan blue exclusion test (Fig. 2J).

3.3. Oregonin reduces the production of ROS by human macrophages

To study the effect of oregonin on ROS formation, we analyzed H_2O_2 levels in lysates from human LPS-activated macrophages after 24 h incubation with or without oregonin. After incubation with oregonin, H_2O_2 levels were significantly decreased (Fig. 3A). Taken together, these results suggest that oregonin reduces the production of ROS in human proinflammatory macrophages. We also investigated the role of oregonin on the production of superoxide anion by PMA-activated macrophages. The addition of 50 μmol/L oregonin to macrophages led to reduction of the CL signal (Fig. 3B).

3.4. Oregonin induces the expression of antioxidant-related genes in LPS-exposed primary human macrophages

To elucidate the mechanisms involved in the antioxidant effects of oregonin on macrophages, we compared the levels of oxidative stress and antioxidant defense genes in LPS-exposed primary human macrophages (Table 1). The transcriptional profiling of the 84 oxidative stress related genes was obtained by the Human Oxidative stress and Antioxidant defense RT2 Profiler PCR array and was used to produce a hierarchical clustering scheme of 25 significantly regulated genes (Fig. 4). We found that oregonin increased the mRNA expression levels of *HMOX1* by 95-fold and *HSPA1A* by 35-fold (Table 1). Oregonin also increased the mRNA expression levels of several antioxidant-related genes (*GPX4*, *GSS*, *GSTP1*, *SOD1*, *SQSTM1*, *SRXN1*, *TXN*, *and TXNRD1*) and reduced the mRNA expression levels of several important oxidative stress responsive genes (*CCL5*, *MPO*, *NCF1*, *PDLIM1*, *PTGS1*, *PTGS2*, *and PREX1*).

4. Discussion

In this study, we characterized the effects of oregonin, a natural diarylheptanoid isolated from *A. incana*, on inflammatory responses in human macrophages. We found that oregonin caused decreased macrophage lipid accumulation, decreased cytokine and ROS production.

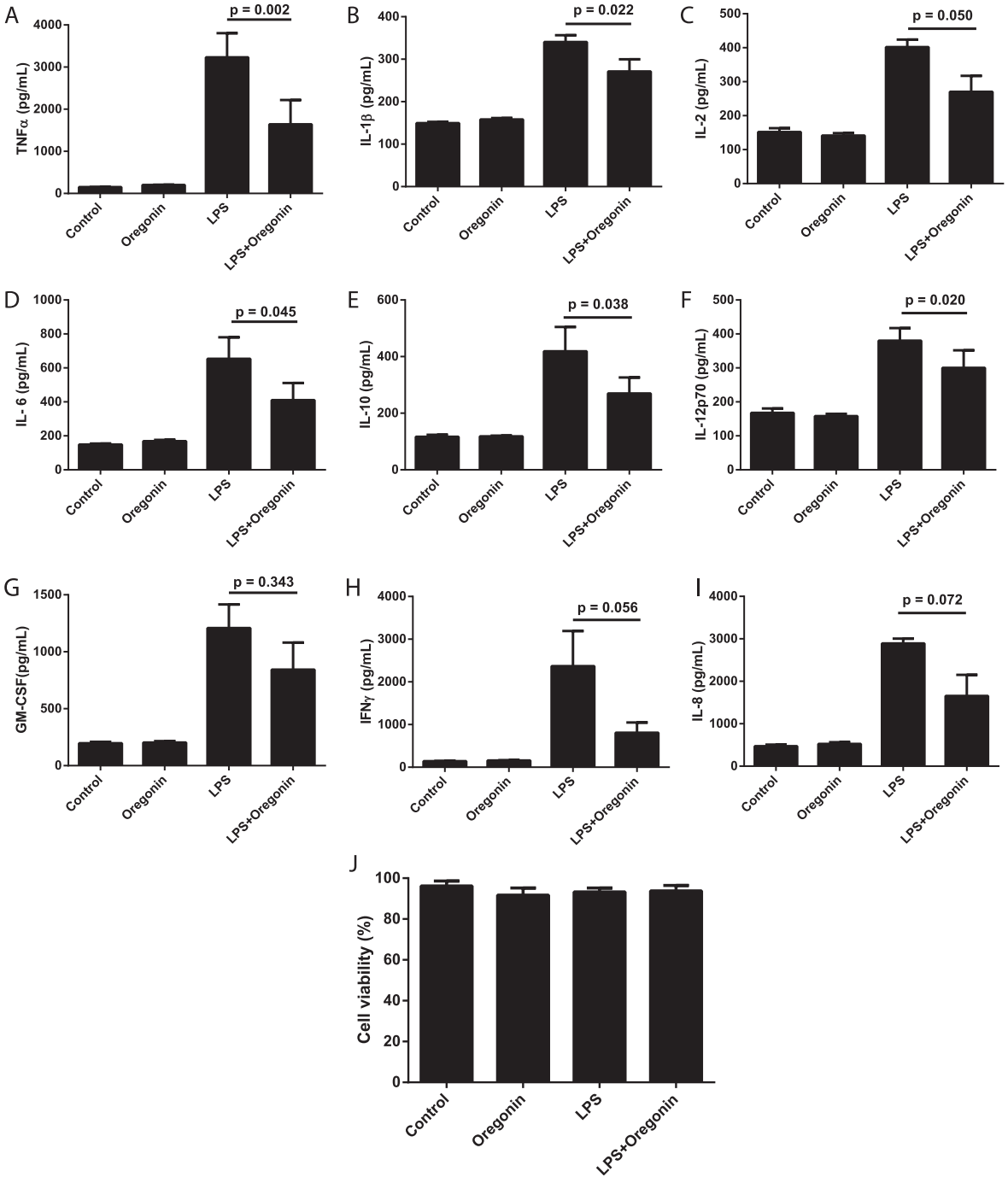

Fig. 2. Decreased cytokine production in oregonin-treated primary human macrophages. Primary human macrophages were incubated with or without 1 μg/mL LPS and 50 μmol/L oregonin. Cytokine concentrations in supernatants were measured after 24 h. Quantification of secreted cytokines in supernatants: **A)** TNF-α; **B)** IL-1β; **C)** IL-2; **D)** IL6; **E)** IL-10; **F)** IL12p70; **G)** GM-CSF; **H)** IFN-γ; and **I)** IL-8. **J)** Exposure to 50 μmol/L oregonin did not affect cell viability (measured by the trypan blue exclusion test). Each bar indicates the mean ± SEM of primary human macrophage cultures (n = 4 different donors).

Uptake of atherogenic lipoproteins in macrophages is a key event in atherogenesis, and macrophage-derived foam cells promotes lesion progression and the majority of acute complications of atherosclerotic disease [7]. We found that oregonin caused markedly decreased cellular lipid accumulation in ischemic human macrophages, suggesting that oregonin reduces foam cell formation. These observations support previously reported findings about treatment of hypercholesterolemic rats with oregonin leads to reduction of blood lipids as well as reduced content of lipid droplets in hepatocytes [14].

The inflammatory response in leukocytes involves increased production of lipid droplets, and that the enzymes involved in the biosynthesis of eicosanoids, which are mediators of inflammation, are localized around the lipid droplets [18]. Thus, the accumulation of lipids in macrophages may be part of the inflammatory response [19].

Further analyses of human macrophages revealed that oregonin significantly decreased the secretion of the proinflammatory cytokines TNF-α, IL-1β, IL-2, IL-6, IL-10, and IL-12p70. Lipid-filled macrophages in lesions generally have the properties of

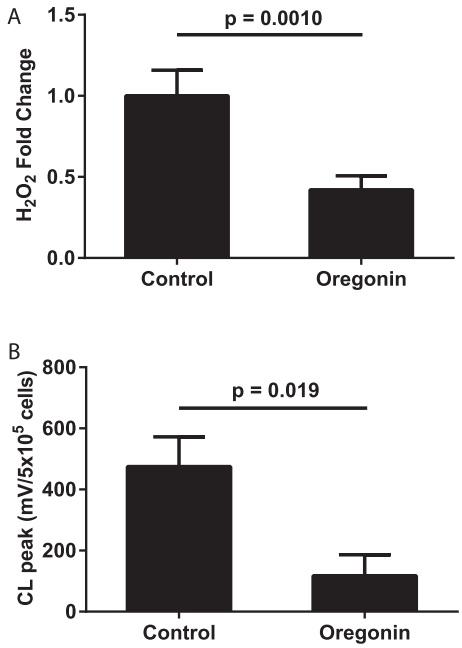

Fig. 3. Oregonin decreases ROS production by primary human macrophages. **A)** Primary human macrophages were incubated for 24 h with or without 1 μg/mL LPS (Sigma), in the absence or presence of 50 μmol/L oregonin. Oregonin decreases H_2O_2 production in human macrophages. Data are presented as fold changes in mean H_2O_2 levels ± SEM in primary human macrophages (n = 4 different donors). **B)** Superoxide anion production was measured after macrophage stimulation with 5 nmol/L PMA, in the presence of 50 μmol/L oregonin or without oregonin (control). Chemiluminescence was recorded for 20 min. ROS production by macrophages was expressed as maximal peak values ± SEM (in mV) that were produced from 5 × 10^5 viable cells from four macrophage donors.

proinflammatory M1 subtype macrophages and secrete the cytokines TNF-α and IFN-γ. These cytokines cause the activation of macrophages and vascular cells, promote inflammation, and participate in cellular immunity [20]. Oregonin effectively reduced myeloperoxidase (MPO) levels in macrophages. MPO is found in atherosclerotic lesions [21], promotes foam cell formation [22], and impairs cholesterol efflux from macrophages [23]. Our data suggest that the lipid-reducing effect of oregonin is partially mediated by decreased MPO expression.

We also provide evidence for the importance of ROS in the anti-inflammatory response observed in oregonin treated macrophages. The role of ROS in inflammatory processes is unclear and ROS has been described both as a trigger and effector of inflammation [24]. It has been reported that ROS may serve as important signaling messengers and that superoxide anion and hydrogen peroxide are ROS that may act as second messengers to activate inflammation that results in IL-1β production [25]. Previous data showed that extracts from *A. japonica*, including oregonin, had a superoxide radical scavenging effect [26]. Several pathways have been suggested to be involved in the antioxidant activity of oregonin in LPS-activated rabbit macrophages, including the reduction of inducible nitric oxide synthase (iNOS) and the induction of hemeoxygenase-1 [27]. We found no evidence of iNOS activation in oregonin-treated human macrophages. These divergent results may relate to the use of different cell types for oregonin treatment. In the presence of oregonin, both superoxide anion and hydrogen peroxide production is reduced in primary human macrophages, which suggests that oregonin exhibits antioxidant activity that results in decreased cytokine production.

The antioxidant effect of oregonin with reduced levels of ROS in oregonin treated macrophages was consistent with observations from our gene expression analysis. We found that the most strongly induced gene was *Heme oxygenase decycling 1* (*HMOX1*) in oregonin-treated macrophages. HMOX1 has antioxidant, anti-inflammatory, and cytoprotective properties and is a rate-limiting enzyme in heme catabolism [28]. Additionally, we observed increased expression of *Heat shock 70 kDa protein 1A* (*HSP701A*) in oregonin-treated cells. This finding might indicate the induction of a defense mechanism against cytotoxic effects, as HSP90 has been shown to protect cells from ischemic cell injury [29]. Our data suggest that oregonin acts as an indirect antioxidant and exhibits antioxidant effects by inducing cytoprotective compounds and proteins, such as HMOX1, HSP70A1, glutamate-cysteine ligase modifier (GCLM), NAD(P)H quinone oxidoreductase 1(NQO1), soluble superoxide dismutase (SOD1), and thioredoxin reductase (TXNRD1) [30].

We also showed increased regulation of the genes for the glutathione metabolic process in oregonin treated macrophages, several genes in the glutathione pathway are induced by oregonin, including *GCLM*, *glutathione transferase* (*GSTP1*), *glutathione synthase* (*GSS*), and *glutathione peroxidase* (*GPX 4*). Glutathione has a predominant role in the regulation of the intracellular redox state and protects cells and tissues from oxidative injury [31]. Glutamate-cysteine ligase (GCL) is the first rate-limiting enzyme in glutathione synthesis. This enzyme consists of a catalytic subunit and a modifier subunit, GCLM. Low levels of *GCLM* mRNA in human macrophages resulting from mutations in *GCLM* have been associated with susceptibility to myocardial infarction [32].

Under conditions of oxidative stress the chemokine CCL5 plays a critical role as macrophage activator in atherosclerosis and myocardial infarction [33]. We showed that the oxidative stress responsive gene *CCL5* is downregulated by oregonin. CCL5 are increased by oxidative stress and are tightly connected to a

Table 1
Up–Down Regulation of antioxidative and oxidative stress genes expressed in lipopolysaccharide-exposed macrophages from three different macrophage donors treated with oregonin and without as control.

Gene symbol	Gene name	Fold change	P value
HMOX1	Heme oxygenase (decycling) 1	94.95	0.00006
HSPA1A	Heat shock 70 kDa protein 1A	35.98	0.04650
GCLM	Glutamate-cysteine ligase modifier subunit	16.46	0.00095
NQO1	NAD(P)H dehydrogenase quinone 1	7.30	0.00096
SOD1	Superoxide dismutase 1. soluble	5.88	0.00135
TXNRD1	Thioredoxin reductase 1	4.47	0.03491
SRXN1	Sulfiredoxin 1	4.37	0.00041
BNIP3	BCL2/adenovirus E1B 19 kDa interacting protein 3	3.58	0.00791
SQSTM1	Sequestosome 1	2.97	0.00114
GSTP1	Glutathione S-transferase pi 1	2.40	0.05178
CCS	Copper chaperone for superoxide dismutase	2.09	0.04436
GSS	Glutathione synthetase	1.82	0.00355
GPX4	Glutathione peroxidase 4	1.70	0.06241
SIRT2	Sirtuin 2	1.67	0.04083
Downregulated genes			
CCL5	Chemokine (C–C motif) ligand 5	−20.44	0.06660
NCF1	Neutrophil cytosolic factor 1	−15.61	0.00307
MPO	Myeloperoxidase	−14.26	0.02365
PDLIM1	PDZ and LIM domain 1	−8.77	0.00937
PTGS1	Prostaglandin-endoperoxide synthase 1 (prostaglandinG/H synthase and cyclooxygenase)	−7.75	0.00002
GPX1	Glutathione peroxidase 1	−3.15	0.00466
CYBB	Cytochrome b-245 beta polypeptide	−2.46	0.02231
SOD2	Superoxide dismutase 2, mitochondrial	−2.28	0.02226
MSRA	Methionine sulfoxide reductase A	−2.01	0.03896
GTF2I	General transcription factor IIi	−1.74	0.03112
PDRX3	Peroxiredoxin 3	−1.64	0.00278

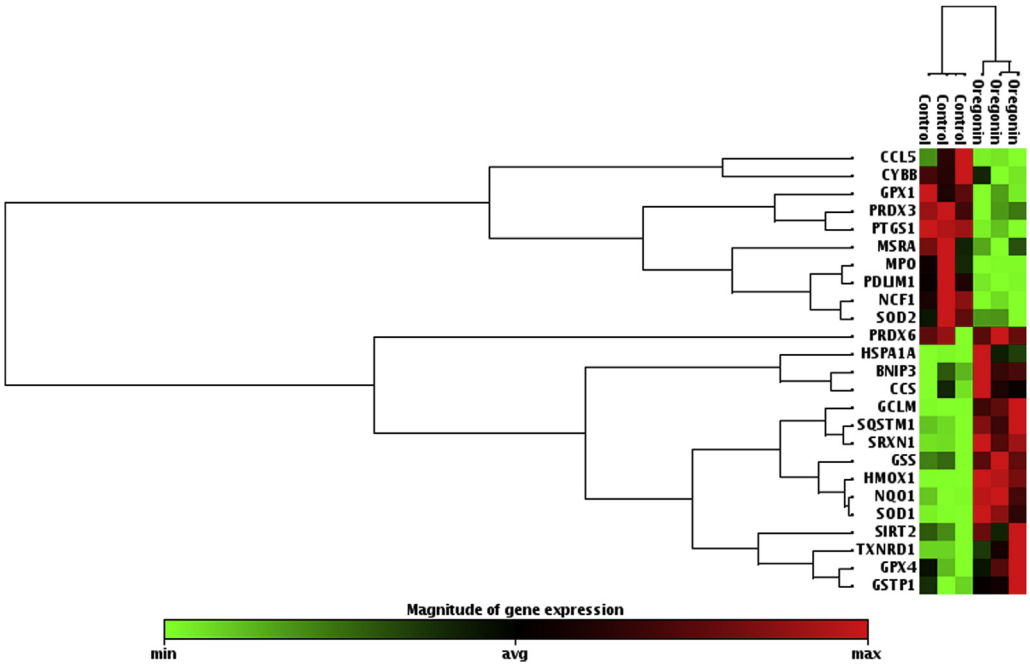

Fig. 4. Hierarchical clustering of oxidative stress and antioxidant defense genes. Each row represents a single gene; each column represents lipopolysaccharide-exposed macrophages from three different macrophage donors treated with oregonin and without (control).

signaling pathway involving NF-κB activation whereas specific inhibition of ROS prevented NF-κB activation [34]. Furthermore, expression of *NCF1* the 47 kDa cytosolic subunit of neutrophil NADPH oxidase is decreased by oregonin. The respiratory burst induced via activation of *NCF1* is one of the major mechanisms of ROS formation in macrophages and is activated to produce superoxide anion [7]. Our chemiluminescence data show a decreased superoxide production in oregonin exposed macrophages which may be explained by the reduced *NCF1*.

Our findings indicate that oregonin from *A. incana* decreases inflammation, lipid accumulation, and ROS production in primary human macrophages. Oxidative stress, derived from excessive and persistent production of ROS and a possible decrease in antioxidant defenses, leads to increased amounts of inflammatory mediators upon inflammatory challenge. Mechanisms underlying the effects of oregonin on inflammation may be via modulation of oxidative stress as we detect increased expression of protective genes and reduced expression of oxidative stress responsive genes. We propose that the anti-inflammatory bioactivity of oregonin is mediated through the HMOX1-regulated antioxidant defense pathway and that oregonin may be a useful candidate therapeutic molecule to use to treat oxidative stress-related diseases.

Author contributions

Conceived and designed the experiments: AL, LUM, and LMH. Contributed reagents/materials and analytic tools: JK, GT, TD, and LMH. Performed the experiments: AL, LUM, CU, and LMH. Analyzed the data: AL, LUM, CU, and LMH. All authors contributed to the writing and editing this manuscript.

Conflict of interest

The manuscript authors confirm that they have no commercial associations that pose a conflict of interest in connection with publication of this article.

Acknowledgments

This work was supported by the Swedish Research Council (521-2013-3588), the Swedish Heart-Lung Foundation (20130231), and Laboratory Medicine, Sahlgrenska University Hospital, Gothenburg Sweden.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.01.161.

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